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13. ABSTRACT (Maximum 200 Words)

One of the most frequent alterations in breast cancer is deregulation of the p53 tumor suppression signaling pathways. The **tumor suppressor p53** is a sequence-specific transcription factor that is activated in response to various cellular stresses. It has been predicted that there are hundreds of consensus p53 binding sites present in the human genome. To date, numerous seminal **p53 target genes** have been identified, leading to the further elucidation of the role of p53 in tumor suppression. However, only a subset of p53 target genes has been identified to date, and characterization of p53 signaling pathways in their entirety is not yet complete. The goal of this study is to further define and characterize such pathways through the identification of novel genes that are directly regulated by p53. Using chromatin immunoprecipitation followed by a yeast selection system we have isolated over 100 genomic DNA fragments that contain **novel p53 binding sites**. The new DNA fragments obtained have been mapped to various regions of the human genome, and putative novel p53 target genes have been identified, validated, and characterization is ongoing. This research will lead to a more complete understanding of p53-regulated signaling pathways in **mammary epithelial cells**.

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INTRODUCTION

As stated in my original grant proposal, the overall goal of my project was to explore the pivotal role that the transcription factor p53 plays in suppressing neoplastic transformation of the normal mammary epithelium. Specifically, I proposed to study p53 signaling pathways in primary mammary epithelial cells in response to oncogenic stress as compared to genotoxic stress. As outlined below and in the previous annual summary the working hypothesis has been changed from looking at both oncogenic stress and genotoxic stress, to only genotoxic stress. The current hypothesis is **p53 protein levels in the cell and the heterogeneity of the p53 consensus DNA binding site contained within downstream target genes dictate p53 target gene selectivity in mammary epithelial cells after genotoxic stress.**

BODY

The hypothesis for the proposed studies involving an oncogenic stimulus was based on observations from other laboratories indicating that overexpression of oncogenes (such as myc and ras) would activate p53. ErbB2 is an oncogene found to be overexpressed in 20-30% of primary human breast tumors (1, 2), and this was the oncogene of choice for our studies due to its physiological relevance. However, upon ectopic expression of ErbB2 in primary human mammary epithelial cells, we found that p53 protein levels decreased and target gene induction was not seen. Our data was subsequently supported by a paper from Mien-Chie Hung's group in which they showed in NIH3T3 cells that overexpression of ErbB2 induces p53 ubiquitination via phosphorylation of Mdm2 by Akt (3). Mdm2 is an E3 ubiquitin ligase protein that negatively regulates p53 through ubiquitination and subsequent degradation (4, 5). Phosphorylation of Mdm2 by Akt leads to Mdm2 activation and decreased protein levels of p53 (3). The original intent of this project was to compare the p53 binding sites and target genes isolated in response to oncogenic stress versus genotoxic stress. As mentioned in the previous annual summary, due to the above-mentioned oncogene inconsistencies, I reprioritized my project to pursue the proposed genotoxic stress studies as a primary objective. Thus, I will be reporting primarily on the work that I've completed for my original Statement of Work with regards to genotoxic stress. Throughout this past year, I have focused on Task 2 of the grant being that the parts of Task 1 that were still relevant were completed by the end of the first year of training.

Task 1. To determine the kinetics of p53 binding to select target genes, quantify p53 protein levels, and assess the phosphorylation and acetylation status of p53 *in vivo* in human mammary epithelial cells after oncogenic stimulation.

a. Obtain normal human mammary epithelial cells and mammary epithelial cell lines to be used in the following experiments and verify p53 status (Months 1-3). **COMPLETED**

- A purification process for primary human mammary epithelial cells (HMECs) from reduction mammoplasty breast tissue as well as the growth conditions for these cells have been optimized. Experiments have been completed that determined the p53 status in each sample. All samples obtained to date contain functional p53 in response to genotoxic stress.

b-e. Due to the inconsistencies experienced with regards to the activation of p53 by the ErbB2 protein (discussed above) we have not been able to proceed with tasks b-e. Instead, we have

focused on the Task 2 objectives. **DISCONTINUED**

Task 2. To "trap" p53 *in vivo* at consensus DNA binding sites after genotoxic stress, and analyze: (i) the heterogeneity of the sites to which p53 is bound compared to the canonical binding site, (ii) the affinity of p53 for a select number of the DNA binding sites recovered, and (iii) the genetic loci and adjacent coding region for novel sites.

a. Create libraries of DNA fragments to which p53 binds in response to genotoxic stress by cloning the fragments recovered from chromatin immunoprecipitation into a yeast reporter vector. (Months 6-12). **COMPLETED**

- To date three libraries from different cell cultures have been generated. The first library generated was from MCF-10A cells, an immortal but nontransformed mammary epithelial cell line; the second library was generated from the primary human mammary epithelial cells (HMECs), and the third library was generated from the MCF-7 breast cancer cell line. All cells used contain functional p53. The p53-activating treatment was 350 nM adriamycin for 6 h. Adriamycin (doxorubicin) is a commonly used breast cancer chemotherapeutic agent.

b-c. Use a functional yeast selection system to identify p53 DNA-binding sites that promote transcriptional activation of a reporter gene, and sequence the DNA fragments recovered from the yeast screen (Months 12-18). **COMPLETED**

- The yeast screen of all 3 libraries generated above has been completed. We have identified more than 100 novel p53 binding sites from the human genome. In addition, we have recovered binding sites of previously identified p53 target genes, including p21 and Mdm2 (Table 1).

d. Test p53-binding DNA fragments identified in (b) in a mammalian cell reporter assay (Months 12-24). **SUPERSEDED/COMPLETED**

- At this point in the study we felt it was more important to analyze the *in vivo* regulation of the genes that the binding sites were regulating instead of using less informative *in vitro* assays to measure transcriptional activation potential. To that end we performed chromatin immunoprecipitation using a p53 specific antibody followed by PCR of the p53 binding site containing DNA fragments recovered to test for *in vivo* binding in ***multiple*** cell lines (for example see Figure 1).

e. Compare the characteristics and heterogeneity of the newly identified p53 binding sites isolated from mammary epithelial cells subjected to oncogenic stress versus genotoxic stress (Months 24-36). **DISCONTINUED**

- Being that the oncogenic arm of this project was discontinued, we cannot address Task 2e.

f. Test the affinity of p53 for the newly identified binding sites by employing an *in vitro* binding assay (Month 12-24). **COMPLETED**

- A select number of DNA fragments isolated from the screen that contained p53 binding sites and resided near genes that we found to be p53-regulated were tested for *in vitro* DNA binding. By using mutational analysis we proceeded to show that p53 is binding specifically to the putative binding site found within the DNA fragments isolated (for example see Figure 2).

g. Use genome sequence resources to identify candidate genes that may be directly regulated by p53 binding to the novel sites identified, and verify p53-dependent gene transactivation of these candidate genes through Northern analysis (Months 12-36). **IN PROGRESS**

- We define a candidate gene as one that is located within 20 kb from the genomic location of the fragment that was isolated by chromatin immunoprecipitation. Candidate gene selection has been performed on all 3 libraries. We have decided that Northern analysis is too time-intensive and instead have changed the validation technique to real-time PCR, which is quicker and more sensitive.

- In addition to the MCF-10A-Neo and MCF-10A-E6 cell lines that were generated earlier in this study (see previous annual summary report), additional primary cells and cell lines were used to validate regulation of putative target genes by p53. These cell lines include the HIp53 cell line, a derivative of the H1299 p53 null lung carcinoma cell line that contains ponasterone-inducible p53, and the isogenic colon carcinoma cell lines HCT116, either p53 null or wild type. The primary cells used to validate the putative target genes were human mammary epithelial cells (HMECs) isolated from reduction mammoplasty breast tissue, and human epidermal keratinocytes (HKs) isolated from the foreskins of newborn males.

- All candidate genes have been identified and validation of their p53-dependent regulation is ongoing (Table 1). One such candidate gene, endothelin-2 (EDN-2), has been actively pursued, and the characterization of this gene in relation to p53 signaling pathways is almost complete. To date, we've used real-time PCR to validate EDN-2, and we are currently performing experiments to determine its biological function (Figure 3). In addition to EDN-2, other candidate target genes are also being pursued.

KEY RESEARCH ACCOMPLISHMENTS

- Completed the generation of all 3 libraries (MCF-10A, HMEC, and MCF-7).
- Completed the yeast screen for all 3 libraries generated.
- Completed *in vitro* DNA-binding assays to test relative affinity of p53 for select novel p53 binding sites recovered.
- Performed chromatin precipitation in multiple cell lines to validate p53 binding to regions of DNA containing the novel p53 binding sites identified.
- Validated endothelin-2 as a novel p53 target gene.

REPORTABLE OUTCOMES

- Poster presentation: American Association for Cancer Research
  Advances in Breast Cancer Research
  Huntington Beach, CA
  October 8-12, 2003
  title: Identification of Novel p53 Target Genes in Mammary Epithelial Cells

- Poster presentation: Vanderbilt-Ingram Cancer Center-Meharry Medical School Retreat
  Nashville, TN
  June 2004
  title: p53 Signaling in Mammary Epithelial Cells

- Publication: Jamie Hearnes, Deborah Mays, Lucy Tang, Kristy Schavolt, and Jennifer Pietenpol. Using Chromatin Immunoprecipitation to Identify Direct Transcription Factor Gene Targets: Endothelin-2 is a p53-regulated Gene. SUBMITTED

CONCLUSIONS

The overall goal of this research project is to further elucidate p53 signaling pathways in mammary epithelial cells by identifying novel p53 target genes. The results described above show that novel p53 binding sites are being isolated using our experimental approach, and these binding sites are linked to physiologically relevant p53 target genes. The new p53 binding sites obtained have been mapped to various regions of the human genome, and numerous putative p53 target genes have been identified and validated, including endothelin-2, and characterization is ongoing. Although the goals of the research project have been reprioritized with regards to the oncogenic and genotoxic studies, the data obtained from the genotoxic stress portion is itself very valuable and will lead to a more complete understanding of p53-regulated signaling pathways in mammary epithelial cells and how deregulation of these pathways can lead to breast cancer.

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APPENDIX

Table 1. Representative summary of recovered p53 consensus binding sites and known or putative target genes

Clone #	Chr.	Gene name	Gene description	p53 binding site	Match	Location
1	6	CDKN1A	Cyclin dependent kinase inhibitor 1A(p21, Cip1)	cAACATGTTg-0-GGACATGTTC	18	5'
2	6	CDKN1A	Cyclin dependent kinase inhibitor 1A(p21, Cip1)	AGACTgGgCa-0-tGtCTgGgCa	12	5'
3	12	MDM2	Murine double minute 2, p53 binding protein	GGtCAAGTTC-0-AGACAcGTTC	18	intron
4	11	DDB2	Damage-specific DNA binding protein 2 (p48)	GAACAAGCCC-1-GGGCATGTTT	20	exon
5	1	EDN-2	Endothelin-2	ctGCAAGCCC-0-GGGCATGCCC	18	intron
6	4	PDGFC	Platelet-derived growth factor isoform C	GGtCATGTTC-0-AGACTTGCCC	19	intron
7	21	LOC388833	Predicted	AGACATGTCC-0-tGGCATGCCC	19	5'
8	1	PLA2G2D	Phospholipase A2, group IID	AGGCATGTgC-0-AAACATGCCC	19	5'
		PLA2G2F	Phospholipase A2, group IIF			5'
9	12	RBM19	RNA binding motif protein 19	GtACATGTCa-0-GGGCATGTTg	17	intron
10	3	SMARCC1	SWI/SNF related, matrix-associated, actin dependentreg. of chromatin	GGGCATGaTg-0-GctCATGCCT	16	intron
11	1	PTPN14	Protein tyrosine phosphatase, non-receptor type 14	GGACATGTTg-0-cAACATGTTT	18	intron
12	3	PPP2R3A	Protein phosphatase 2, regulatory subunit B", alpha	AGACATGTCT-2-AGACATGCCC	20	intron
13	10	VTI1A	Vesicle transport through interaction with t-SNAREs homolog 1A	cAACATGTCT-0-tGGCATGTTC	18	intron
14	15	FLJ32800	Predicted	AGGCATGTCT-0-GGACATGTTT	20	5'
		MDS009	X 009 protein			intron
15	16	ANKRD11	Ankyrin repeat domain protein 11	AGACATGaCC-0-tGGCATGTTC	18	intron
		LOC400562	Predicted			3'
		LOC390751	Predicted			5'
16	9	C9ORF88	Predicted	tGGCAAGCag-0-AGGCTTGTTT	17	intron
17	6	MICA	MHC class I polypeptide-related sequence A	GGGCATGTCT-0-GGACAAGTCT	20	5'
18	6	TMEM30A	Transmembrane protein 30A	GGACTTGCTT-0-GGGCTTGTCC	20	intron
19	13	BCMS	B-cell neoplastia-associated gene with multiple splicing	GGACATGCCC-0-GGACAAGCCT	20	intron
20	12	FLJ25323	Predicted	tGGCATGTCC-0-ccACATGTCC	17	5'
21	14	AKAP6	A Kinase anchor protein 6	AGACATGCCC-0-AAACATGTCT	20	intron
22	10	ANKRD2	Ankyrin repeat domain 2	tGACAAGTCT-0-GGGCTTGCTC	19	5'
		UBTD1	Ubiquitin domain containing 1			intron
23	1	H1160	n/a	AGGCATGCCC-0-GGGCATGTCT	20	n/a
24	5	H1026	n/a	GGGCATGTTg-0-GGGCATGTCC	19	n/a
25	13	H1524	n/a	AGGCATGTCT-0-GGGCAAGTTa	19	n/a

Table 1. A representative sample of p53 binding sites that were recovered from the libraries are listed along with the corresponding candidate target genes that could potentially be p53-regulated. Also listed are the chromosomal locations of the binding sites, the # of bp matching the 20 bp consensus p53 binding site, and the location of the binding sites with regards to the candidate genes listed.

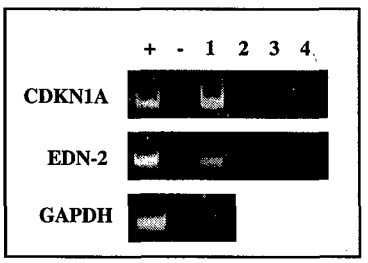

Figure 1. Representative samples of PCR from chromatin immunoprecipitations used to validate p53 binding to sequences of DNA containing putative or known p53 binding sites. CDKN1A (p21) is an example of a known p53 target gene binding site that was recovered from the library. EDN-2 (endothelin-2) is an example of a new p53 binding site (and corresponding gene) recovered. GAPDH was used as a negative control. (+) is PCR from genomic DNA, used as a positive control. (–) is a negative control with no DNA. Lane 1: PCR of p53 immunoprecipitated DNA from cells treated with adriamycin and crosslinked. Lane 2: Identical to Lane 1 except immunoprecipitated with cyclin B1 instead of p53 as a negative control. Lane 3: Identical to Lane 1 except not crosslinked. Lane 4: Identical to lane 1 except the cells were not treated with adriamycin.

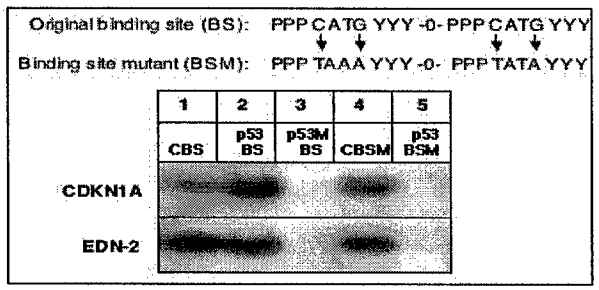

Figure 2. *In vitro* DNA binding assays show p53 binds specifically to the putative p53 binding site found in the endothelin-2 gene. In this assay wildtype and mutant p53 were used in combination with original or mutated p21 and endothelin-2 p53 binding sites to determine specificity of p53 binding. The mutant binding sites were generated by changing the core C nucleotides to T, and the core G nucleotides to A. The C and G core nucleotides are the primary requirements of the consensus p53 binding site. Lane 1: 5% of the radiolabeled original binding site (CBS) DNA used in the assay was loaded as a control for relative comparison of binding. Lane 2: Binding of wildtype p53 to the original binding sites (BS) of p21 or endothelin-2. Lane 3: Binding of mutant p53 to the original binding sites (BS) of p21 or endothelin-2. Lane 4: 5% of the radiolabeled binding site mutant (CBSM) DNA added to the assay was loaded as a control for relative comparison of binding. Lane 5: Binding of wildtype p53 to the p21 and endothelin-2 binding site mutants.

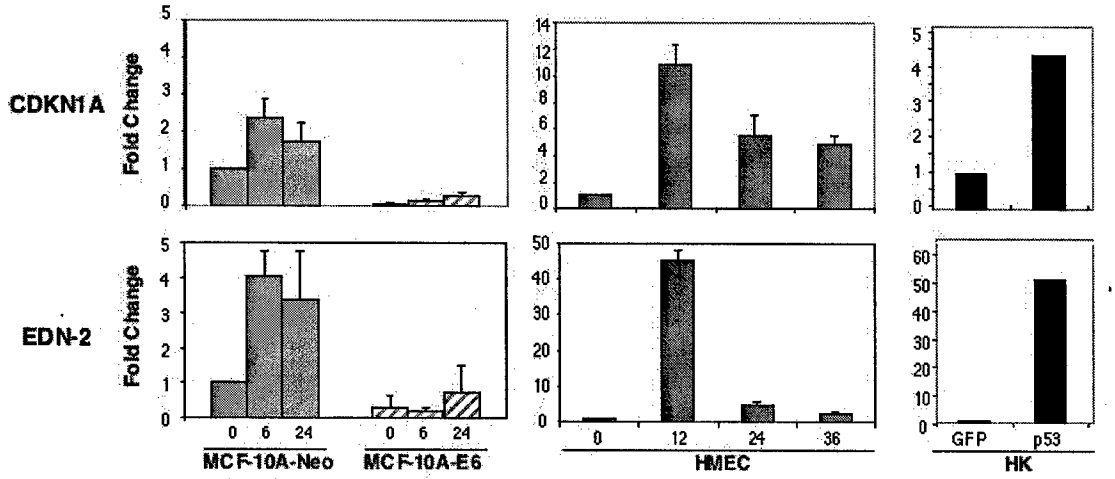

Figure 3. Real-time PCR analysis of the expression of CDKN1A and EDN-2 in multiple cell lines after p53 activation due to ectopic expression of p53 or cell damage. The MCF-10A-Neo cells containing functional p53, MCF-10A-E6 cells containing nonfunctional p53, and primary Human Mammary Epithelial Cells (HMEC) containing functional p53 were treated with 350 nM adriamycin for the indicated timepoints (h), and then harvested total RNA was reverse transcribed and analyzed using the BioRad iCycler Real-time PCR machine. The CDKN1A and EDN-2 gene levels were normalized to GAPDH and the fold change calculated. The primary Human Epidermal Keratinocyte (HK) cells were adenovirally infected with either GFP or p53 for 30 h and the harvested RNA was treated as with the MCF-10A and HMEC samples.